

# Expression of the CTCF-paralogous cancer-testis gene, brother of the regulator of imprinted sites (BORIS), is regulated by three alternative promoters modulated by CpG methylation and by CTCF and p53 transcription factors

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## ABSTRACT

**BORIS**, like other members of the ‘cancer/testis antigen’ family, is normally expressed in testicular germ cells and repressed in somatic cells, but is aberrantly activated in cancers. To understand regulatory mechanisms governing human **BORIS** expression, we characterized its 5′-flanking region. Using 5′ RACE, we identified three promoters, designated A, B and C, corresponding to transcription start sites at −1447, −899 and −658 bp upstream of the first ATG. Alternative promoter usage generated at least five alternatively spliced **BORIS** mRNAs with different half-lives determined by varying 5′-UTRs. In normal testis, **BORIS** is transcribed from all three promoters, but 84% of the 30 cancer cell lines tested used only promoter(s) A and/or C while the others utilized primarily promoters B and C. The differences in promoter usage between normal and cancer cells suggested that they were subject to differential regulation. We found that DNA methylation and functional p53 contributes to the negative regulation of each promoter. Moreover, reduction of CTCF in normally **BORIS**-negative human fibroblasts resulted in derepression of **BORIS** promoters. These results provide a mechanistic basis for

understanding cancer-related associations between haploinsufficiency of **CTCF** and **BORIS** derepression, and between the lack of functional p53 and aberrant activation of **BORIS**.

## INTRODUCTION

**BORIS** (Brother of the Regulator of Imprinted Sites), also designated **CTCF**L (CTCF-like), is the mammalian paralog of a highly conserved (1,2), multi-functional chromatin factor encoded by a candidate tumor suppressor gene, **CTCF** (3–6). Loukinov *et al.* (4,7) showed that in humans and mice, these two genes encode polypeptides of similar size that share a centrally positioned nearly identical DNA-binding domain (DBD). The DBD is composed of 11 Zn-fingers (11ZF), including ten of the classic DNA-binding C2H2-class and one (ZF 11) of the C2HC-class capable of binding both single-strand DNA and RNA (8). It is noteworthy that the 11 ZF DBD regions in **BORIS** and **CTCF** are encoded by genomic sequences, which display an accurate duplication of a region containing all ZF-coding exons of the **CTCF** gene from an early mammal. The accuracy of this duplication in the human genome was maintained to the extent that genomic nucleotide similarities between **CTCF** and **BORIS** at individual intron–exon junctions approach 100% identity at the single nucleotide level (7).

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By virtue of this shared DNA-recognition domain, BORIS can bind specifically to the same DNA target sequences that interact with CTCF. However, the consequences of BORIS or CTCF bound to the same site would be expected to be different. This is because the amino- and carboxy-termini of BORIS and CTCF are totally unrelated such that cofactors associated with one would not partner with the other. Thus, there would be different functional outcomes depending on which one of the two paralogs occupies a CTCF/BORIS-11ZF-binding site (9,10).

Once bound to DNA, CTCF may function as a versatile component of transcriptional chromatin insulators, hormone-dependent silencers and promoter repressors or activators (3–6). CTCF-binding sites have been mapped in differentially methylated domains of imprinting control regions (ICR) that regulate a parent-of-origin-dependent mono-allelic expression within clusters of imprinted genes: *IGF2/H19* (11–13), *Rasgrf-1* (14) *DLK1/GTL2* (15), *AWT1/WT1-AS* (16) and *KvDMR* (17) loci. In addition, several functionally distinct, important, methylation-sensitive CTCF/BORIS-binding sites involved in random and/or imprinted X-chromosome inactivation (XCI) choice have been mapped at the X-inactivation center (Xic) (18–22).

Finally, a novel class of methylation-insensitive, but CpG-containing CTCF/BORIS-target sites was found and characterized in promoter regions of genes encoding the X-linked cancer-testis antigens (CTA) *MAGE-A1*, *NY-ESO-1* (9,23) and *SPAN-X* (24). Promoters of CTA genes are methylated and repressed in normal somatic cells that express *CTCF* and not *BORIS*, but are specifically demethylated and activated in testicular germ cells and in cancer cells that express *BORIS* (9,23). Vatolin *et al.* (9) and Hong *et al.* (23) have documented co-expression of *BORIS* with other CTA genes in the majority of cancer cell lines and primary breast, prostate, colorectal and lung tumors tested. They also showed that conditionally expressed BORIS can outcompete CTCF *in vitro* and *in vivo* for binding to normally methylated 11ZF-target sites present in the silenced *MAGE-A1* and *NY-ESO-1* promoters. In addition, within three days of BORIS expression in normal cells, they observed both demethylation and activation of these two genes. It was also demonstrated that while CTCF is bound to the repressed/methylated *MAGE-A1* and *NY-ESO-1* promoters in normal human fibroblasts, activation of the same promoters in cancer cell lines is associated with *in vivo* occupancy by BORIS of methylation-insensitive 11ZF-target sites. Moreover, 12 other CTA genes that are normally co-expressed in testis with *BORIS* but not in somatic cells with *CTCF* were activated by ectopic expression of *BORIS* in normal primary human fibroblasts. In contrast, promoters of genes regulated by methylation but not normally co-expressed with BORIS in male germ cells failed to respond to ectopic expression of BORIS (9). Similar findings on the important roles of CTCF/BORIS-binding sites in regulation of the germ cell specific *ALF* and *SPAN-X* genes, have been reported by DeJong (25) and Larionov (24) laboratories, respectively.

Importantly, during male germ cell development, CTA-expressing cells are also engaged in the process of re-establishing paternal gene imprinting marks. This suggests that binding of BORIS in place of CTCF to shared targets, may be responsible for two effects. The first is activation of CTA gene promoters in testis. The second is re-setting of paternal ICR marks by remethylation in mature differentiating testicular germ cells after erasure of all gene imprinting marks in primordial germ cells (7,10). Indeed, data from a recent publication have provided strong support for the concept of BORIS-mediated epigenetic regulation of paternal ICR re-methylation (10).

Taken together, these results strongly support the hypotheses that CTCF and BORIS act successively to govern epigenetic states in normal male germ cell development, while rivalry in binding to the same spectrum of DNA sites caused by aberrant activation of BORIS in somatic cells may be associated with cancer (4,7).

While the mechanisms governing regulation of *CTCF* by its promoter have been explored by Klenova *et al.* (26), data on *BORIS* promoter regulation has not been described. This prompted us to investigate patterns of *BORIS* expression in normal testicular germ cells, in cancer cell lines, and in primary tumor tissues. We characterized the 5'-flanking noncoding region of the gene with expected regulatory (promoter) function. We mapped three alternative promoters producing five alternatively spliced 5'-UTR and analyzed promoter usage in different cell types and tissues. We also showed that activity of these promoters is negatively regulated by DNA methylation and by CTCF and p53.

## MATERIALS AND METHODS

### Cell lines and tissue samples

A total of 293 human embryonic kidney cells; normal human dermal fibroblasts (NHDF) (Cambrex, Rockland, MD, USA), normal foreskin fibroblasts BJ and 31 tumor cell lines (Table 1) were used in this study. The majority of tumor cell lines were maintained in RPMI 1640 supplemented with 10% heat inactivated FBS, 1% PSN Antibiotic Mixture (Invitrogen, Carlsbad, CA, USA) and 0.5% fungizone (Invitrogen). The 293, HeLa and Co115 cell lines were grown in DMEM with 10% heat inactivated FBS, 1% PSN and 0.5% fungizone. Normal cells were grown in the media recommended by the ATCC and NHDF in the media recommended by Cambrex. K562 cells stably transfected with the temperature-sensitive p53<sup>Val135</sup> mutant, which adopts a wild-type conformation at 32°C, were previously described (27). The p53<sup>-/-</sup> H1299 parental cell line and the H1299 p53wt cell line (28) were a generous gift from Dr Peter M. Chumakov (Department of Molecular Genetics Lerner Research Institute, Cleveland, Ohio, USA). Demethylation studies were performed with NHDF treated with 1 μM 5-aza-2'-deoxycytidine (5-aza-dC) (Sigma-Aldrich, St. Louis, MO, USA). Cells were harvested at different times after treatment and medium containing 5-aza-dC was replaced with the fresh medium every 48 h.

**Table 1.** Cell lines used for screening of BORIS expression from the three different promoters

Group	Cell line	Cancer type	BORIS expression	PrA%	PrB%	PrC%	Methylation MS-SSCP <sup>a</sup>		P53 <sup>b</sup>
							PrB	PrC	
A	SNB-19	CNS <sup>c</sup>	++	95	0	5	M	M	Mut
	786-0	Kidney	++	84	0	16	M	M	Mut
	A498	Kidney	+	65	0	35	M	M	Mut
	ACHN	Kidney	+	78	0	22	M	M	Mut
	CCRF-CEM	Leukemia	+	66	0	34	U/M	U/M	Mut
	RPMI-8226	Leukemia	+	61	0	39	U/M	U/M	Mut
	HOP-92	Lung	+	93	0	7	M	M	Mut
	SK-MEL-2	Melanoma	++	68	0	32	M	M	WT
	OVCAR-3	Ovary	++	83	0	17	M	M	WT
A/C	NCCIT	Testis	+	96	1	3	M	M	Mut
	HS578T	Breast	++	41	0	59	M	M	WT
	MCF7	Breast	++	43	0	57	M	M	Mut
	MDA-MB-435	Breast	+	50	1	49	M	M	Mut
	HCT-116	Colon	++	47	0	53	M	M	Mut
	SW-620	Colon	+	51	0	49	M	M	Mut
	SN12C	Kidney	++	53	0	47	M	M	Mut
	NCI-H522	Lung	+	48	0	52	M	M	Mut
	SK-OV-3	Ovary	++	49	0	51	M	M	WT
C	MDA-MB-231	Breast	+	21	0	79	M	M	Mut
	SNB-75	CNS	++	36	0	64	M	M	WT
	HCT-15	Colon	++	33	0	67	M	M	Mut
	HT29	Colon	+++	37	0	63	M	M	WT
	HL-60	Leukemia	+	39	0	61	M	M	Mut
	MOLT-4	Leukemia	++	25	0	74	M	M	Mut
	SR	Leukemia	+++	31	0	69	M	M	Mut
	NCI-H322	Lung	+++	13	0	87	M	M	Mut
B	COLO-205	Colon	++	3	73	24	U/M	U/M	?
	K-562	Leukemia	+++	3	80	18	U	U	Mut
	MM-S1	Leukemia	+++	2	85	13	U	U	Mut
	NCI/ARD-RES	Ovary	+++	1	52	47	U	U	Del
	OVCAR-8	Ovary	+++	0	96	4	U	U	?
Normal cells	BJ	Fibroblast	—	—	—	—	M	M	n/a
	NHDF	Fibroblast	—	—	—	—	M	M	n/a

<sup>a</sup>U = unmethylated; M = methylated; U/M = partially methylated.<sup>b</sup>Mut = mutation; WT = wild type; Del = deletion (35).<sup>c</sup>CNS = Central nervous system.

Normal and tumor tissues were obtained from the Tissue Bank of the Institute of Pathology of Lausanne. Twenty-four human normal tissue samples from bone marrow, bladder, heart, kidney, skin, colon, testis and ovary and 26 tumor tissue samples from bladder, breast, colon, kidney, lung, testis, ovary and endometrium were analyzed. All tissue samples were examined histologically by a pathologist (R.B.). The use of human tissue samples during this study was in accordance to the guidelines of the ethical committee of the Medical Faculty of Lausanne (Switzerland).

#### Analysis of mRNA stability of BORIS alternative 5'UTRs

The mRNA half-life of BORIS was determined in K562 and Ovar-8 cell lines treated with Actinomycin D (ActD). Briefly, Ovar-8 cells were grown in 6-well tissue culture plates for 24 h to achieve ~80% confluency. Next, cells were treated for 10 min, 30 min, 1, 2, 4, 6, 8, 12, and 24 h with 15 µg/ml of ActD dissolved in DMSO. Total RNA was extracted before and after ActD treatment, and mRNA levels were quantitated by real-time RT-PCR (qPCR). For each sample, the amount of BORIS mRNA

was quantified relative to 1 µg of total RNA. First strand cDNA was synthesized using 1 µg of total RNA (DNase-treated) and alternative 5' UTRs of BORIS mRNA were amplified using primers specific for each isoform (Table 2). Serial 10-fold dilutions (from 300 000 to 30 molecules) of cloned DNA for each alternative 5' UTRs were used as a reference for the standard curve calculation. All qPCRs were performed using the SYBR Green fluorogenic dye and data were analyzed using ABI system software.

RNA degradation curves were obtained by setting as 100% the maximum level of mRNA expression at Time 0 before treatment with ActD. mRNA levels determined at times following Time 0 are expressed as a percentage of the maximum value. The half-lives of alternatively spliced 5' UTR mRNAs was obtained from the logarithmically transformed best-fit line by linear regression analysis (29).

#### RT-PCR and northern blot analyses

RNAs were extracted from cultured cells using the TRIzol LS Reagent (Invitrogen, Basel, Switzerland). One-step RT-PCR was performed with 200 ng of total RNA in 20 µl. BORIS expression was screened with the

**Table 2.** Primers and probes used in real-time PCR

Name	Primers sequences	Probe
CTCF	FW 5'-TGACACAGTCATAGCCCGAAAA-3' REV 5'-TGCCTTGCTCAATATAGGAATGC-3'	6FAMTGATTGGGTGTCCACTTGCGAAAGC-MGB
BORIS-MC	FW 5'-CCCATTGTGCCACCATCA-3' REV 5'-AGCATGCAAGTTGCGCATAT-3'	6FAMACGGAAAAGCGACCTAC-MGB
BORIS promA	FW 5'-CTTACTTCCCCCGGGT-3' REV 5'-GCCTTGGGGTTGAAGTGGA-3'	6FAMCTCCTCCCTTCCTCA-MGB
BORIS promB or BORIS	FW 5'-GCAGAGCCACAAGCCAAAG-3' REV 5'-ATCTCAGTGGCTGCCATAATGACT-3'	6FAMAGTGGGCCGAGCAT-MGB
BORIS promC or BORIS C1	FW 5'-CCCTTCTCCCCCTATGGA-3' REV 5'-CCATAATGACTTGGCCTGTTTG-3'	6FAMACCGCTTGCTTATTT-MGB
BORIS A1	FW 5'-TCCCTTCCTCATCCACTTCAA-3' REV 5'-GCTCAGAAAGGACAGAGATCTCAGT-3'	SYBR Green method
BORIS A2	FW 5'-CATCCACTTCAACCCCAAGC-3' REV 5'-CTTTGGCTTGTGGGCTCTG-3'	SYBR Green method
BORIS A3	FW 5'-GCTCTCCTCCTCTCCTTATCCAC-3' REV 5'-CCATACAGGGCACTGGGAGAC-3'	SYBR Green method
hMYC-G	FW 5'-GTGCGGGAGCCAGTGAAGT-3' REV 5'-AAGATCCCAGCTCCTCAGCC-3'	SYBR Green method
hMYC-N	FW 5'-GGCTCTGTGAGGAGGCAAGGTG-3' REV 5'-GCTCTCTATTTGGAGTGGCGGG-3'	SYBR Green method
CTCF ChIP PrA	FW 5'-CTCCTTATCCATTACCCACCACC-3' REV 5'-CAGTATCTCAGTGCTCCTGTGG-3'	SYBR Green method
CTCF ChIP PrB	fw 5'-CCCTGCCCCACAGTACAT-3' rev 5'-TTTTCCGCTCCGCGC-3'	SYBR Green method
CTCF ChIP PrC	fw 5'-GGCCAGTCCCGGTCAAG-3' rev 5'-AGCATGGGCTGTTCTGGG-3'	SYBR Green method

primers RT-A3 5'-AAGCCGCGAACGGAGACGAAG-3' and RT-B3 5'-ACGCCTTCATCCACTTCCTCTTT-3'. Northern blot analyses were performed as described previously (30).

### Reverse transcription and qPCR

RNA was extracted from cultured cells as described below from tumor cell lines (Table 1). Total RNA (3–4 µg) was converted to cDNA using ThermoScript reverse transcriptase kit (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions.

qPCR analyses were performed using the TaqMan® Universal PCR Master Mix or the PowerSYBR® Green PCR Master Mix (Applied Biosystems, Foster city, CA, USA) and the Applied Biosystems 7900HT qPCR system. For the analyses of *BORIS* transcripts expressed from promoter A, primers were designed in the first noncoding exon nearest to promoter A + 1 start site. For the analyses of expression from promoter B, primers were engineered to span the first noncoding and coding exons. For the analyses of expression from promoter C, primers were selected by using the unique sequence of the first coding exon utilized only for promoter C. Primers and probes were selected using Primer Express program, and optimal experimental conditions were established for each set.

Sequences of the primers and probes are summarized in Table 2. Primer/probe mixtures for human glyceraldehyde-3-phosphate dehydrogenase (HS99999905\_m1) and

**Table 3.** Real-time PCR standard curve coordinates and ranges

Plasmid	Curve range (copy number)	Standard curves	
		Slope	R2
GAPDH	30–300 000	−3.31 + 0.01	0.9955 + 0.02
BORIS Prom A	30–300 000	−3.51 + 0.12	0.9941 + 0.0078
BORIS Prom B	30–300 000	−3.17 + 0.02	0.9945 + 0.004
BORIS Prom C	30–300 000	−3.33 + 0.03	0.9943 + 0.0003

p53 (HS00153349\_m1) were purchased as Predeveloped Assay (Applied Biosystems, Foster City, CA, USA). Data were analyzed by comparative Ct method and normalized to the untreated control.

The RNA copy number generated from each promoter was determined using a standard curve following the ABI protocols. To determine transcript copy number, standard curves were generated for each primer pair using 10-fold serial dilutions of linearized plasmids containing known copy numbers of the target PCR products. The threshold cycle of each dilution was determined and plotted against the log value of the cDNA copy number. Transcript numbers for each experimental sample were interpolated by its detection threshold value using the appropriate standard curve. Expression levels were normalized to that of the housekeeping gene, *GAPDH*. Standard curve coordinates and ranges are shown in Table 3.



### Identification of transcription initiation sites using RNA ligase-mediated rapid amplification of 5' cDNA ends (5' RLM-RACE)

The GeneRacer system (Invitrogen, Carlsbad, CA, USA), based on RNA ligase-mediated and oligo-capping rapid amplification of cDNA, was carried out based on the manufacturer's instructions. The kit ensures the amplification of only full-length transcripts by eliminating truncated messages from the amplification process. Total RNA was dephosphorylated using calf intestinal phosphatase and then decapped to target full-length messenger RNAs. An RNA oligonucleotide was then ligated to the full-length decapped mRNAs. Ligated mRNA was reverse transcribed with a BORIS-specific primer located in the first exon (RACE-EX1 5'-CAGAGGTACGCTCGGCCTCCAAC-3', +159 to +138). Then the 5' cDNA end was amplified by nested PCR using the GC-Rich PCR system (Roche) and with BORIS-specific reversed primers (RACE-EX1N 5'-GGCCTTTTTCGCGCATCAACT-3', +79 to +59; RACE-N 5'-TTGGGGTTGAAGTGGATGAGGAAG-3', +1294 to +1271). Amplified products were separated by electrophoresis on 1% agarose gels and purified on SNAP columns (Invitrogen, Carlsbad, CA, USA). Purified PCR fragments were cloned in a pCR<sup>®</sup>2.1-TOPO<sup>®</sup> vector (Invitrogen), sequenced and analyzed.

### Luciferase reporter vectors and p53 expression vectors

Fragments from the region 5' of *BORIS* were prepared by PCR amplification. The amplification process introduced Asp718I and HindIII sites in the 5' and 3' ends, respectively. All fragments were inserted into the pGL3-basic vector (Promega, Madison, WI, USA) cut with Asp718I and HindIII. Wild type and mutant p53 expression vectors were a generous gift from Dr Peter M. Chumakov.

### Transient transfection and siRNA transfection assays

For luciferase assays, 293 cells were transiently transfected using Eugene 6 (Roche, Germany) according to the manufacturer's instructions. K562 cells were transfected with a Nucleofector device (Amaxa, Inc., Cologne Germany) according to the manufacturer's recommendations with Nucleofector Solution V and program T16 generating the highest transfection efficiency with the lowest mortality. HeLa, NCCIT and OVCAR-3 cells were seeded in 12-well dishes at a concentration of  $3 \times 10^5$  cells/well, one day before transfection. The cells were transiently transfected with the different constructs (0.75 µg/well) with the jetPEI Cationic Polymer Transfection reagent (4 µl/well) (PolyPlus-transfection, Illkirch, France) according to the manufacturer's instructions. All experiments were performed at least three times. The pRL-tk vector (0.25 µg/well) (Promega, Madison, WI, USA) was co-transfected as an internal control for transfection efficiency. Luciferase assays were performed using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA). To compare results, the mean values of relative luciferase activity were used. The expression levels of the different constructs were compared

to the level of the pGL3-control vector containing the firefly luciferase gene under the control of the SV40 early promoter and to the level of the pGL3-basic vector.

SiRNA assays were performed in 6-well plates using the Interferin reagent (PolyPlus-transfection, Illkirch, France) according to the manufacturer's instructions. Stealth<sup>TM</sup> RNAi for CTCF 5'-GCGCUCUAAGAAAGAAGAU UCCUCU-3' was synthesized by Invitrogen (Invitrogen, Carlsbad, CA, USA). Stealth<sup>TM</sup> RNAi Negative Control High GC siRNAs for silencing control was provided by Invitrogen (Invitrogen, Carlsbad, CA, USA). Transfected cells were harvested 48 h post-transfection.

### DNA methylation analysis of promoters B and C

DNA was extracted from frozen tissue or culture cells using the DNeasy tissue kit (Qiagen, Hilden, Germany). Two microgram of DNA were modified in 40 µl of water with sodium bisulfite as previously described (31) or using the EpiTect<sup>®</sup> Bisulfite Kit (Qiagen, Hilden, Germany). After bisulfite modification, PCR on BORIS promoter B was performed with the primers 5'-CCTCCCCCAACCCT ACCTAA-3' and 5'-GTTTTTGGTTTGTGGGTTTT GTT-3' using the master mix (Promega, Madison, WI, USA) with a final concentration of 5% DMSO, under the following PCR conditions: 35 cycles of 94°C for 30 s, 54°C for 45 s and 72°C for 50 s. The first PCR product diluted 1/50 was amplified by semi-nested-PCR with the primers 5'-CACTACCACCTCCACTCTC-3' (+931 to 1950) and 5'-GTTTTTGGTTTGTGGGTTTTGTT-3' (+964 to +980). Each PCR product was analyzed by MS-SSCA and MS-DBA (31,32), as previously described. In the MS-DBA approach, two 3'-end DIG-labeled probes, 5'-AACCCGACGACGACCGAC-3' and 5'-CCAACCC AACACAACCAAC-3', were used to recognize the methylated and the unmethylated DNA, respectively. PCR on BORIS promoter C were performed with primers 5'-ACAAAACCCACAAACCAAAA-3' and 5'-TTTTT GGAGGAGAGTAGGTG-3' using the Platinum PCR supermix (Invitrogen, Carlsbad, CA, USA) with a final concentration of 5% DMSO using the following PCR conditions: 40 cycles at 94°C for 30 s, 52°C for 45 s and 72°C for 50 s. PCR products amplified from NHDF, NCCIT, OVCAR-3, K562 and OVCAR-8 cells were cloned into pCR<sup>®</sup>2.1-TOPO<sup>®</sup> vector (Invitrogen, Carlsbad, CA, USA). Ten clones for each cell lines were sequenced (Genomics Research Facility, Rocky Mountain Laboratories, NIAID/NIH, Montana, USA).

### Electrophoretic mobility shift assay (EMSA)

Fragments containing BORIS promoters A, B and C were synthesized by PCR. EMSA was performed as previously described (33). Briefly, PCR fragments were end labeled using <sup>32</sup>P-γ-ATP and T4 polynucleotide kinase (New England Biolabs). Protein-DNA complexes were allowed to form by incubation for 30 min at ambient temperature in PBS with 5 mM MgCl<sub>2</sub>, 0.1 mM ZnSO<sub>4</sub>, 1 mM dithiothreitol, 0.1% Nonidet P-40, 10% glycerol and poly(dI-dC). Full-length CTCF and the ZF DNA-binding domain of CTCF were translated *in vitro* using the TnT kit (Promega, WI, USA). Protein-DNA complexes were

resolved from unbound DNA probe using a 5% native polyacrylamide gel (PAAG) in 0.5× TBE.

### Chromatin immunoprecipitation assay (ChIP)

Normal fibroblast BJ cells and two cancer cell lines, Ovar-3 and Ovar-8, were used in ChIP assays to examine the *in vivo* binding of CTCF on *BORIS* promoters. We used a ChIP Assay kit (Upstate, Charlottesville, VA, USA) and followed the manufacturers' recommendations. One ChIP reaction used 10 µl of the anti-CTCF monoclonal antibodies previously described (20). The human CTCF-binding site N, a c-MYC insulator site (hMYC-N), was used as positive control and CTCF non-binding site G of c-MYC (hMYC-G) was used as negative control. Immunopurified DNA was used in real-time PCR using primers described in Table 2.

## RESULTS

### Identification of *BORIS* transcription initiation sites

To identify transcription initiation sites, 5' RLM-RACE was performed. The 5' RLM-RACE method has a major advantage over other methods for mapping of transcription start sites (e.g. primer extension, nuclease protection assays or traditional 5' RACE) in that it only detects authentic capped 5' ends of mRNAs. The technique is based on RNA ligase-mediated (RLM-RACE) and oligocapping rapid amplification of cDNA ends, and results in the selective ligation of an RNA oligonucleotide to the 5' ends of decapped mRNA. This method allowed the amplification of only truly full-length transcripts via elimination of truncated messages. The 5' RLM-RACE assays were performed on total RNA extracted from normal testis tissue, and from the NCCIT tumor cell line. Two PCR products (~700 bp and ~1500 bp) were obtained and, after cloning and sequencing, the first two start sites were identified at -658 and -899 bp from the ATG translational start site (Figure 1A). Using the GC-Rich PCR system and nested primers upstream of the first transcription start site, a ~200 bp PCR product was generated. This new site mapped to -1447 bp upstream of the ATG translation start codon (Figure 1A).

### Identification of *BORIS* promoters and their transcriptional activities

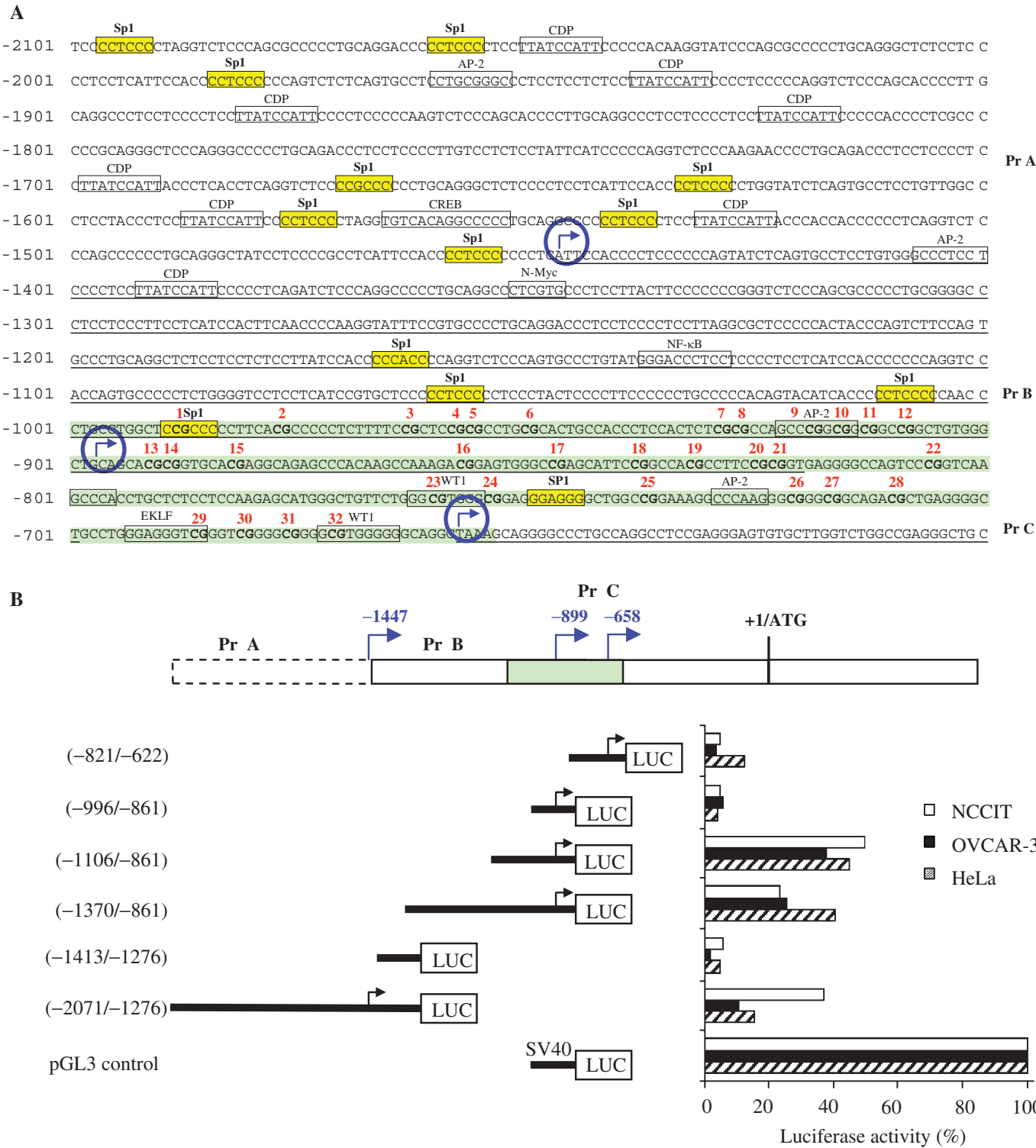
To functionally characterize the three putative promoters, different fragments of the regions upstream of all three transcriptional start sites were cloned into a firefly luciferase expressing vector (Figure 1B). Transfection assays were carried out with the NCCIT, OVCAR-3 and HeLa cell lines. Two reporter constructs were used to study transcriptional regulation by promoter A (Figure 1B). The larger construct (-2071 to -1276) showed substantial transcriptional activity in all three cell lines, whereas the 5' truncated construct (-1413 to -1276) showed only low activity. The activity of promoter A was significantly higher in NCCIT than in OVCAR-3 or HeLa cells. These results suggested that transcriptional activity of promoter A could be cell type-specific. To study

transcriptional regulation by promoter B, three reporter constructs were generated. The construct containing nucleotides -1370 to -861 of the promoter B region produced an activity that was 23–40% of that generated by the SV40 promoter. A construct truncated to contain nucleotides -1106 to -861 was more active, yielding between 38% and 50% of SV40 levels, whereas the transcriptional activity of the smallest construct (-996 to -861) was very low. These results suggest that the sequences located between -1106 and -996 to the ATG translational start site defined the minimal *BORIS* promoter B. Promoter C is located very close to promoter B and is quite small. To study promoter C, we generated a construct containing nucleotides -821 to -622 upstream of the start site. This construct produced relatively low activity in comparison with promoters A and B. The transcriptional activity of promoter C was observed to be higher in HeLa cells corresponding to 18% of SV40 promoter activity, suggesting that promoter C may also be tissue specific (Figure 1B).

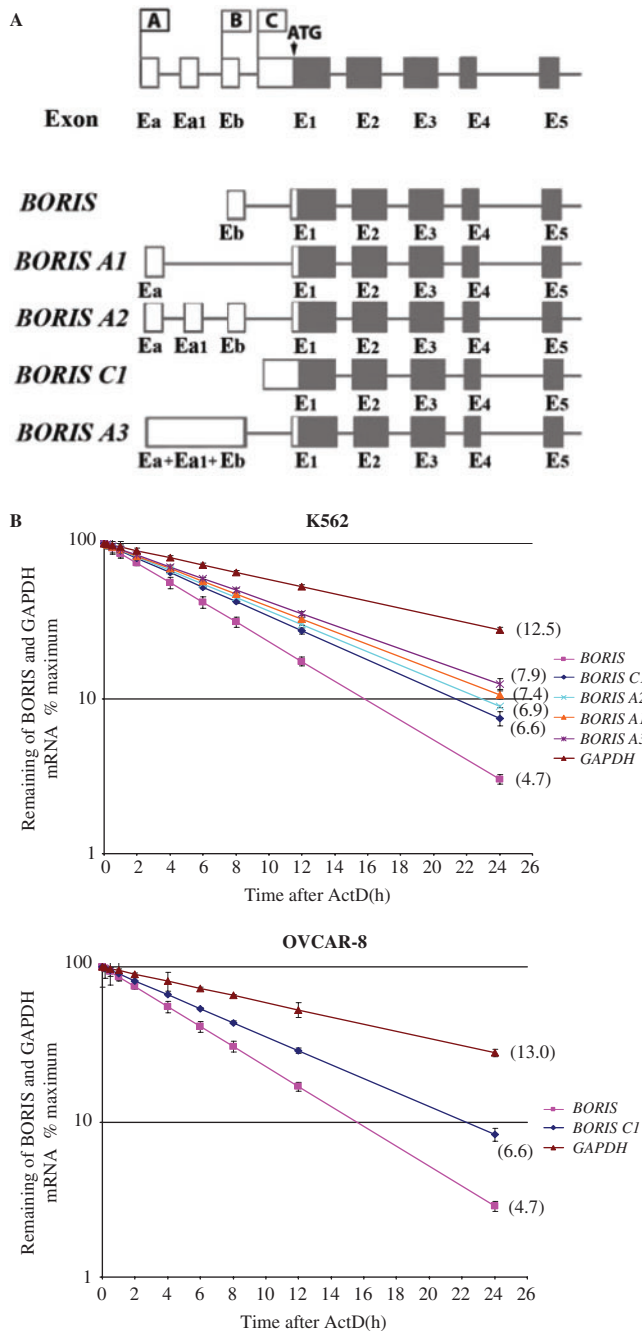
Analyses of the *BORIS* promoter region sequences using the MatInspector program (<http://www.genomatix.de/index.html>) identified several consensus sequences for transcription factor binding sites. In addition, promoters B and C are situated in a CpG island. Promoter B contained putative binding sites for Sp1, AP-2, NF-κB and N-Myc and promoter C contained Sp1, two WT1, AP-2 and EKLf putative binding sites (Figure 1A). Promoter A contained Sp1, AP-2, CREB and a large number of CAAT displacing protein (CDP) putative binding sites.

### Identification of five alternatively spliced 5'-UTRs for *BORIS* and analyses of their stability

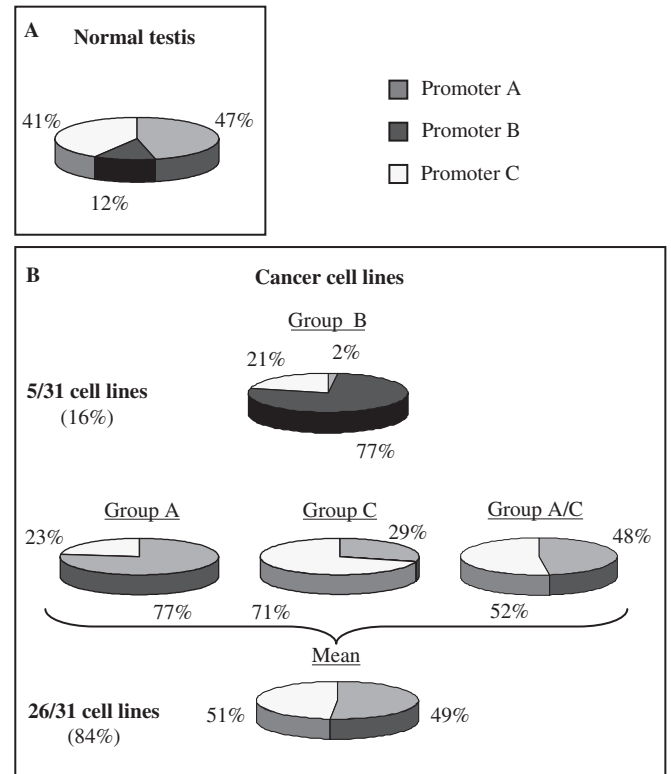
The fact that *BORIS* transcription initiated from three different promoters suggested that there would likely be a series of 5' splice variants. Indeed, we identified five major transcripts that differed in their 5' noncoding regions but encoded the same protein sequence (Figure 2). The *BORIS A1* and *BORIS A3* isoforms are expressed from promoter A and have first 180 and 548 bp noncoding exons Ea and Ea + Ea1 + Eb, respectively that splice directly to the first 530 bp coding exon, E1. The *BORIS A2* splice variant also originates from promoter A, but has two more noncoding exons, Ea1 and Eb, than the *BORIS A1* isoform. The *BORIS* variant that comes from promoter B contains a first 80 bp noncoding exon, Eb, which splices to first 530 bp coding exon, E1. The *BORIS C1* isoform is expressed from promoter C and is transcribed as an unspliced first 1190 bp coding exon (Figure 2A). To investigate whether the alternative 5' UTRs affect mRNA stability, we blocked RNA synthesis by treating cells with ActD. Two cell lines were used in those experiments: K562 cells that express all five alternative 5' UTRs and Ovar-8 cells that express only two of five. As shown in Figure 2 (panel B), the half-lives of *BORIS*, *BORIS A1*, *BORIS A2*, *BORIS A3* and *BORIS C1* transcripts in K562 cells were 4.7, 7.4, 6.9, 7.9 and 6.4 h, respectively, indicating that *BORIS* mRNAs in cultured cells are relatively stable and long-lived. The alternative



**Figure 1.** Identification and activity of three *BORIS* promoters. **(A)** Nucleotide sequence of the 5'-upstream region of the human *BORIS* gene is shown together with a portion of the coding region. Potential binding sites of transcriptional regulatory proteins predicted by the MatInspector program are shown in boxes. Yellow boxes highlight the Sp1 factor. The A in ATG translational start site is designated as nucleotide +1 and does not appear in the figure. The main start sites for transcription, as determined by 5'-RACE, are indicated by encircled blue arrows. The transcribed sequences are underlined. Nucleotides of the CpG island are shaded in green. Red numbers (1-32) represent all CpGs within the CpG island. **(B)** Determination of *BORIS* promoters A, B and C activities by transient expression of luciferase reporter constructs in HeLa, NCCIT and OVCAR-3 cell lines. Left: schematic representation of *BORIS* promoter luciferase reporter constructs. Numbers indicate positions of nucleotides of the 5'-flanking region of the *BORIS* gene, as shown in A. Right: relative luciferase activities compared with the pGL3 control vector activity, which was considered to have 100% activity. The CpG island is shaded in green.



**Figure 2.** Detection of *BORIS* expression from different alternative promoters and stability of *BORIS* alternative transcripts. (A) Unique *BORIS* cDNA sequences attached to promoters A, B and C. To detect *BORIS* expression from different promoters, forward primer was designed on basis of unique cDNA sequence for every promoter. Reverse primer was designed on the basis of *BORIS* coding exon 2 sequence. Expected RT-PCR fragment was 720 bp for *BORIS* promoter A expression, 760 bp for *BORIS* promoter B expression and 810 bp for *BORIS* promoter C expression. All RT-PCR fragments contain splice site allowing distinguishing them from genomic DNA. (B) K562 and Ovar-8 cells were treated with Actinomycin D for various durations to block transcription. Total RNA was extracted at the indicated time points following the addition of actinomycin D. Quantitative real-time PCR amplification were performed as described in Materials and Methods section to determine copy number of each *BORIS* alternative transcript. Each data point represents the average of three amplification reactions. RNA degradation curves were obtained by setting at 100% the maximum of mRNA expression at Time 0 before Act D treatment.



**Figure 3.** *BORIS* expression from different alternative promoters (A, B and C) in normal testis and in multiple types of cancer cell lines by real-time PCR. (A) Relative expression of *BORIS* from the three different promoters in normal testis. (B) Relative expression of *BORIS* from the three different promoters in 31 different tumor cell lines. Tumor cell lines are divided in four groups according to the main promoter usage. Group B represents 16% of all cell lines tested and the *BORIS* expression is mainly originated from promoter B. In the three other groups the activity of *BORIS* comes from either promoter A, either promoter C or from promoters A and C. These three groups represent 84% of all cell lines tested.

transcript expressed from promoter B, *BORIS*, was 1.5-fold less stable than *BORIS* transcripts expressed from promoters A and C. The relative turnover of the three alternative 5' UTRs expressed from promoter A—*BORIS A1*, *BORIS A2* and *BORIS A3*—was comparable and the rates of degradation were approximately 1.2-fold slower than for the *BORIS C1* transcript expressed from promoter C. Similar half-lives of *BORIS* (4.7 h) and *BORIS C1* (6.6 h) were obtained in Ovar-8 cell line (Figure 2B). In both cell lines, *GAPDH* mRNA degradation was similar to published values for that gene (34).

### Analysis of *BORIS* transcription from promoters A, B and C in normal and cancer cells

To gain further insights into the characteristics of promoters A, B and C, we studied their utilization by qPCR in a panel of normal tissues, normal cell lines and cancer cell lines. As expected from previous studies, testis was the only normal tissue to express *BORIS* (data not shown). The relative percent of expression from each promoter was then calculated. Figure 3A shows that in normal testis, expression comes from all three promoters.



The distribution of activity is 47% from promoter A, 12% from promoter B and 41% from promoter C.

The same analysis was performed on 31 cancer cell lines (Table 1, Figure 3B). Only 5 cell lines showed transcriptional activity promoter B and these were assigned to a subset designated as group B. Activity in all the other cell lines came only from promoters A or/and C. These 26 cell lines were assigned to one of three groups, based on their utilization of these two promoters. Group A is comprised of cell lines that expressed *BORIS* primarily from promoter A. Group C includes a series of cell lines that expresses *BORIS* primarily from promoter C. Cell lines in the group designated A/C use both promoters more or less equally. The patterns of promoter utilization in the cancer cell lines are distinct from that observed in normal testis. However, it must be recognized that testis tissue contains germ cells at various stages of spermatogenesis which could utilize the different promoters in a cell type-specific manner or might use all three at each stage. Moreover, based on known features of the 31 cell lines, preferential promoter utilization could not be correlated with tissue origin or with genomic characteristics that might be thought to influence *BORIS* expression—loss of the *CTCF*-containing chromosome 16; gain of chromosome 20 or amplification of 20q13 that contains the *BORIS* locus. In addition, there was no obvious relation between promoter utilization and patterns of p53 expression (Table 1) (35,36). These results showed that all three promoters contribute to *BORIS* transcription, with cancer cells preferentially using promoters A and C.

### Regulation of *BORIS* promoters by CTCF

The fact that expression of *CTCF* and *BORIS* in normal testis is almost mutually exclusive raised the possibility that *CTCF* may negatively regulate *BORIS* transcription. To examine this possibility, we used siRNA to suppress *CTCF* expression in NHDF and then tested the cells for expression of *CTCF* and *BORIS* by qPCR (Figure 4A). We observed that the level of *CTCF* transcript was reduced more than 4-fold in cells expressing the siRNA while the levels of *BORIS* transcripts in the same cells were increased at least 8-fold (Figure 4A). To evaluate the contribution of each promoter to expression of *BORIS* in the siRNA-treated cells, we performed qPCR with primers and probes specific for each of the spliced isoforms. The results showed that in cells expressing the *CTCF*-specific siRNA, expression of *BORIS* from all three promoters was enhanced, but to differing extents—expression from promoter A was increased 8-fold, expression from promoter B was increased 2.5-fold, and expression from promoter C was increased 4.5-fold (Figure 4A). These results indicated that under normal circumstances, *CTCF* contributes to suppression of *BORIS* expression by acting on all three promoters.

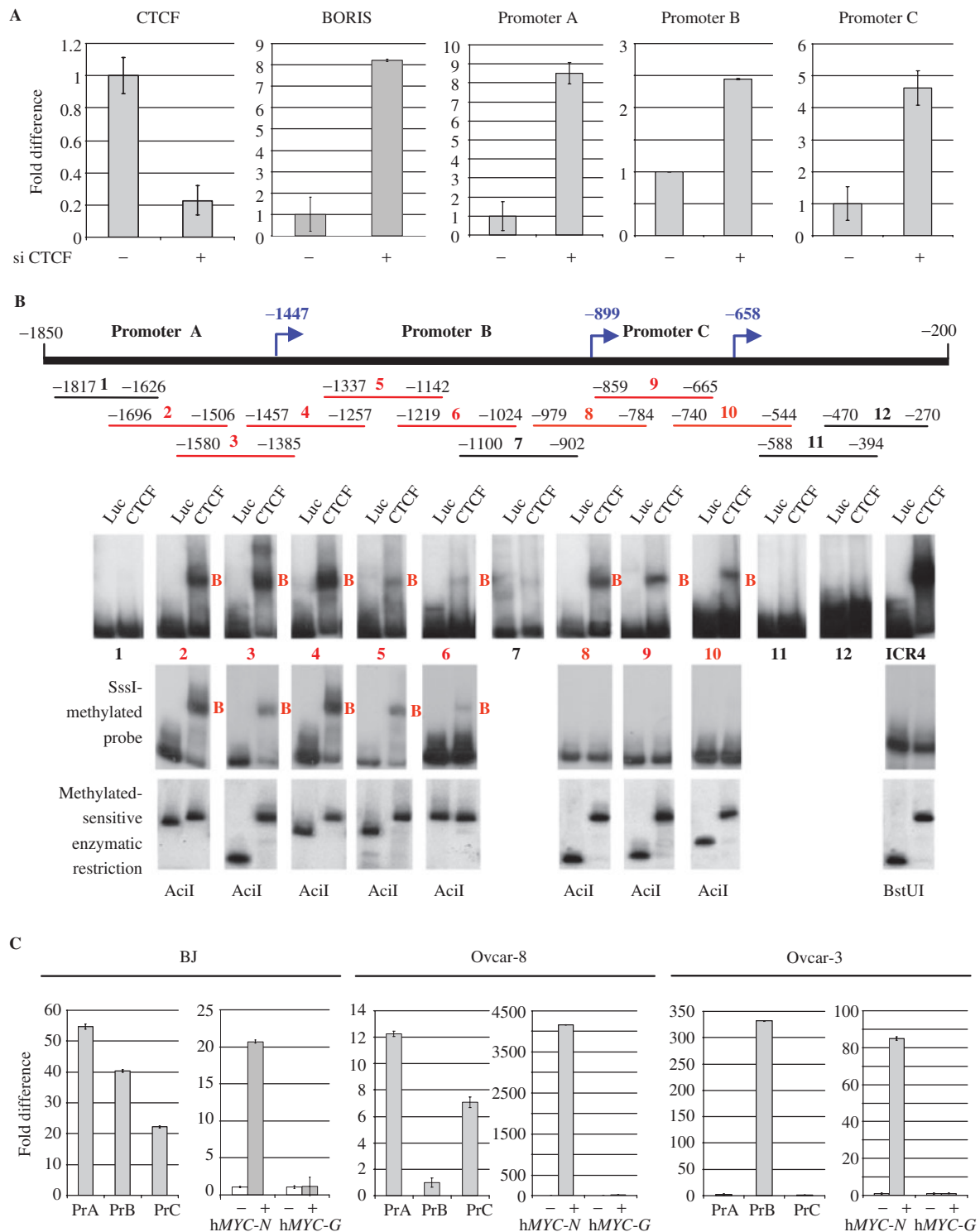
To obtain additional evidence that *CTCF* binds directly to the *BORIS* promoter region, we performed EMSA using recombinant *CTCF* with overlapping probes encompassing *BORIS* promoter sequences. We identified 3 binding sites in promoter A, 3 in promoter B and 2 in promoter C (Figure 4B), supporting the suggestion that

*CTCF* is directly involved in suppressing *BORIS* expression. It is known that *CTCF* binding can be sensitive (11–13,18,20) or insensitive to methylation of its target sites (9,23,24). In order to see if *CTCF* binding within *BORIS* promoters was sensitive to DNA methylation, all fragments positive for *CTCF* binding were methylated *in vitro* using *SssI* DNA methylase. EMSA analysis showed that binding of the *CTCF* 11ZF DNA-binding domain to fragments 2, 3, 4 (promoter A), 5 and 6 (promoter B) was similar whether the fragments were methylated or not (Figure 4B). In contrast, no binding was observed to methylated fragments 8, 9 and 10 that are located in the CpG island shared by promoters B and C. The degree of probe methylation, tested using the methylation-sensitive restriction endonuclease *AcI* for the *BORIS* promoter and *BstUI* for ICR4 as a positive control, showed that the extent of methylation was nearly 100%. This experiment showed that some *CTCF*-binding sites within *BORIS* promoters were methylation-sensitive and others were methylation-insensitive.

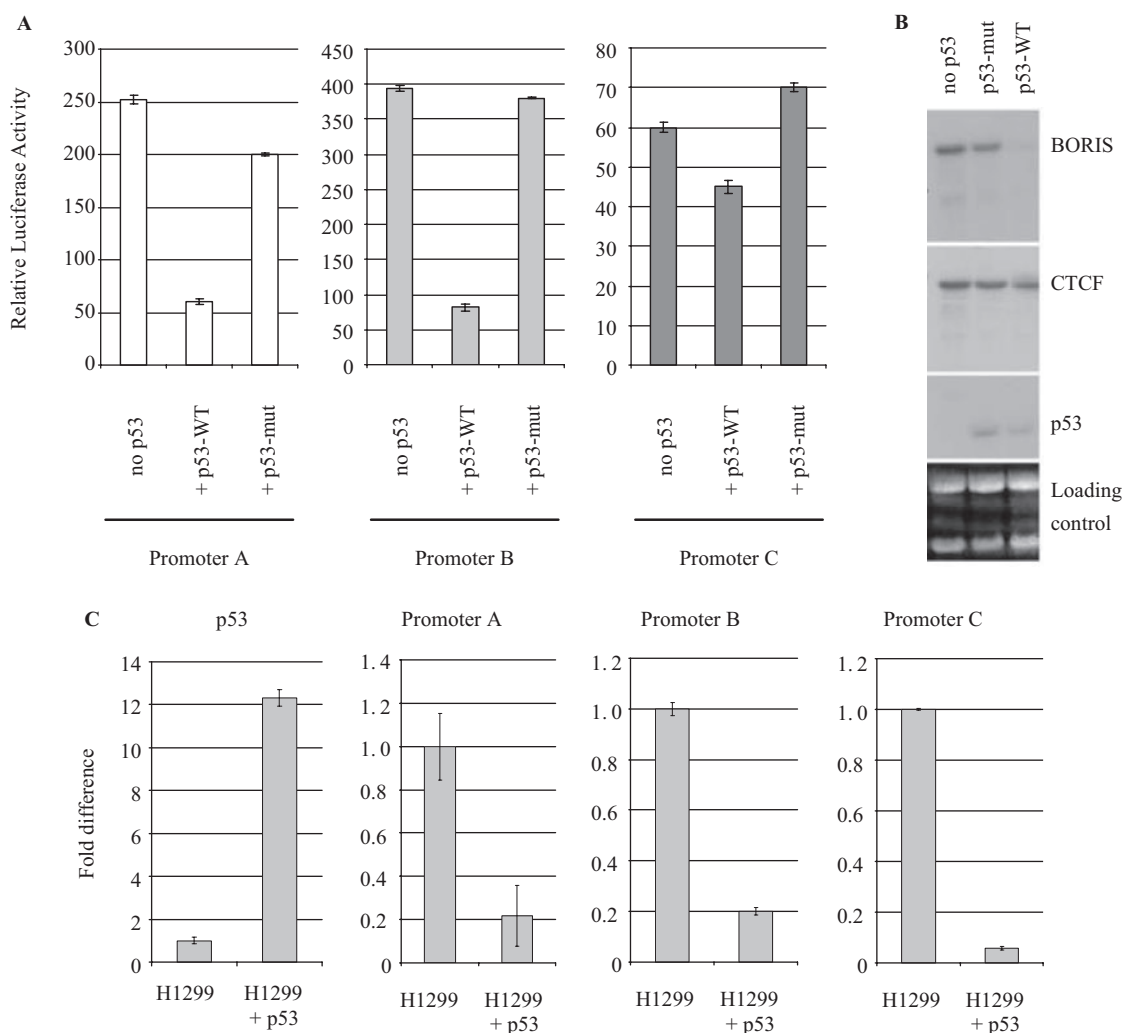
To investigate *CTCF* binding to *BORIS* promoters *in vivo*, we performed ChIP assays in normal BJ fibroblasts, in Ovar-8, using mostly promoter B, and in Ovar-3, using mostly promoters A. We analyzed the promoter regions of *BORIS* and site N from the *c-MYC* locus, a well-characterized *CTCF*-binding site that functions as a 5' chromatin insulator for the locus (3,20,37). Figure 4C showed that site N was occupied by *CTCF* in all three lines while the non-binding target used as negative control was not. In the BJ cell line, occupancy of all three promoters by *CTCF* correlated well with the transcriptionally repressed state of all three in this cell line. In Ovar-8, *CTCF* occupancy was found only on promoter A and C. No *CTCF* binding was found in promoter B, which correlates with expression of *BORIS* coming only from promoter B in this cell line (Table 1). In contrast, *CTCF* binding in Ovar-3 was identified in promoter B but not in promoter C. *CTCF* binding in promoter A was not significant, showing only a 2.2-fold enrichment. These results also correlate with *BORIS* expression coming from promoters A and C, but not from promoter B. Taken together, these results showed that there was an inverse correlation between the extent to which *CTCF* bound to the *BORIS* promoters and the activity of those promoters.

### Regulation of *BORIS* promoters by p53

In our analyses of *BORIS* expression in the 31 cancer cell lines, we noted that the lines expressing *BORIS* at the highest levels were known to have deletions or mutations of *TP53*, the gene encoding the p53 tumor suppressor (data not shown). This prompted us to ask if p53 might be involved in regulating *BORIS* expression. To examine this possibility, we transfected K562 cells that are p53 negative with luciferase reporters for each of the three *BORIS* promoters together with expression plasmids for wild-type or mutant p53 (Figure 5A). The results of these studies showed that the luciferase activity of all three *BORIS* promoters was significantly reduced in cells transfected with wild-type p53 versus mutant p53. With wild-type p53,



**Figure 4.** Regulation of *BORIS* expression by CTCF. **(A)** Real-time PCR analyses of RNA extracted from NHDF (Normal Human Dermal Fibroblasts) treated with siRNA against *CTCF* show the decrease of *CTCF* expression and the increase of *BORIS* expression coming from the three promoters at different levels. Data were analyzed by comparative Ct method and normalized to the untreated control. Results are represented for each set of primers used in real-time PCR. **(B)** Schematic map of the overlapping fragments used as EMSA probes for *in vitro* detection of CTCF-binding sites and results of EMSA analyses showing binding of CTCF within promoters A, B and C in red (first panel). Results of EMSA analyses of SssI-methylated probes showing insensitivity of CTCF binding in promoter A and B, and methylation sensitive sites in promoter B and C within the CpG island (second panel). The third panel showed the methylation-sensitive enzymatic restriction of probes used in methylation-sensitive EMSA. **(C)** CTCF *in vivo* occupancy on *BORIS* promoters via ChIP in normal fibroblasts BJ, Ovar-8 and Ovar-3 cell lines. Real-time PCR analysis of the fold difference for the presence of DNA with the CTCF-binding site in the input chromatin versus in the CTCF chromatin fraction is obtained by ChIP. The fold difference between immunoprecipitated DNA with specific CTCF antibody or rabbit serum (used as negative control) is represented for each set of primers. hMYC-N primers are used as positive control for good enrichment after ChIP and c-MYC non-binding site G is used as control for the specificity of the enrichment. Controls are represented in the right graph for each cell lines.



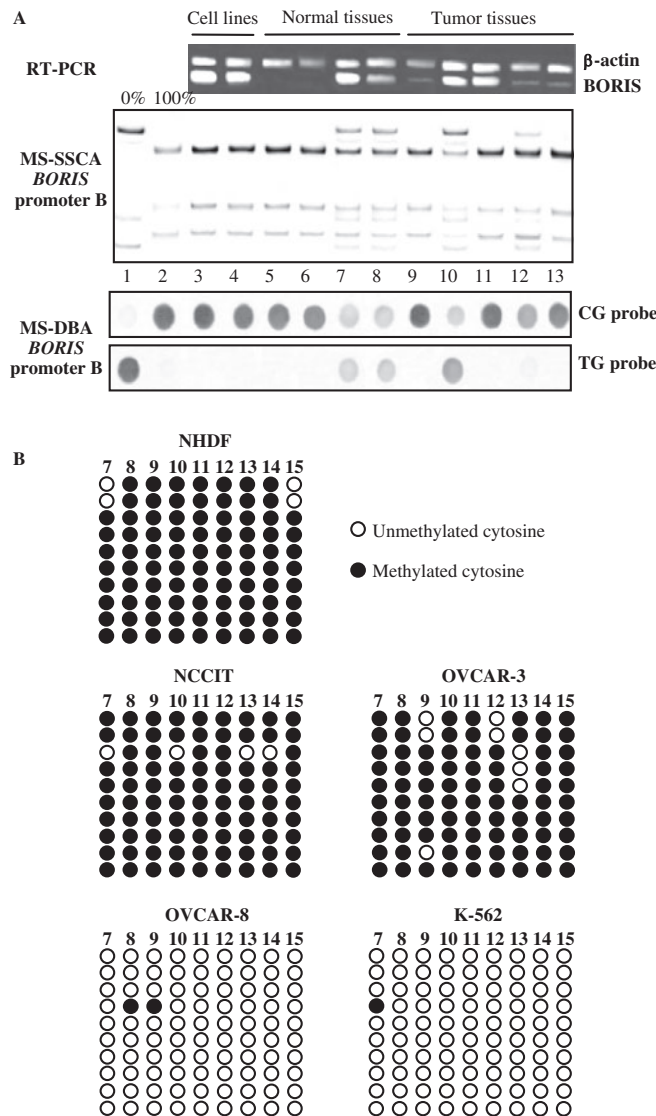
**Figure 5.** p53 effect on *BORIS* promoters A, B and C activities. (A) Luciferase assays were performed by transient transfection of reporter constructs and p53 wild-type (WT) or mutant (mut) expression vectors, in the p53<sup>-/-</sup> K562 cell line. (B) Northern blot showing the expression of *BORIS*, *CTCF* and *p53* in the K562 p53 thermo-inducible cell line. (C) Real-time PCR on RNA extracted from p53<sup>-/-</sup> H1299 parental cells or H1299 cells expressing p53 wild type. Real-time PCR data were analyzed using the comparative Ct method and normalized to the non-induced cells.

the activity of promoters A and B was downregulated about 3.3- and 4.6-fold, respectively, and the activity of promoter C was repressed about 1.5-fold. We then performed northern blot analysis of K562 cells transfected with a vector encoding temperature-sensitive alleles of wild-type and mutant p53. The results showed that over-expression of the mutant p53 had no effect on the levels of either *BORIS* or *CTCF* transcripts. In contrast, activation of wild-type p53 was associated with a great reduction in expression of *BORIS* while expression of *CTCF* was unaffected (Figure 5B). To further examine the impact of p53 on *BORIS* expression *in vivo*, we compared p53-negative H1299 parental cells with H1299 cells stably infected with a virus expressing wild-type p53 (28). The results (Figure 5C) showed that the level of p53 expression was 12-fold higher in the virus-infected H1299 cells than in the parental cells. Analyses of *CTCF* transcripts showed that they were unchanged (data not shown). In contrast, p53 had a profound effect on each of the

three *BORIS* promoters. Expression from promoters A and B was reduced 4.6- and 4.9-fold, respectively, while that from promoter C was down 17-fold.

#### Regulation of *BORIS* promoters by DNA methylation

To further analyze the molecular mechanisms involved in regulating the activity of the *BORIS* promoters, we examined the effect of CpG methylation. The fact that promoters B and C co-localize within a CpG island raised the possibility that their activities might be affected by methylation (Figure 1A). DNA samples from 2 cell lines, 24 human normal tissue samples and 26 tumor tissue samples were extracted and analyzed by methylation-sensitive single-strand conformation analysis (MS-SSCA) and methylation-sensitive dot blot assay (MS-DBA) after sodium bisulfite modification (31,32). The CpG island was found to be completely methylated in samples of all normal tissues with the exception of testis, the tissue in which *BORIS* is normally expressed. Studies of the CpG



**Figure 6.** Analyses of DNA methylation of *BORIS* promoter B. (A) *BORIS* expression and methylation patterns of *BORIS* promoter B in human tissues and cell lines. Lanes 1–2: MS-SSCA and MS-DBA unmethylated and fully methylated controls respectively, obtained from plasmids containing *BORIS* promoter B sequences; lanes 3–4: NCCIT and OVCAR-3 cell lines; lanes 5–8: normal tissues, respectively colon, skin, and two testis; lanes 9–13: tumor tissues, respectively bladder, testis, ovary, breast, colon. *BORIS* mRNA was detected by RT-PCR, with  $\beta$ -actine as internal control. (B) Genomic bisulfite sequencing of 16/32 CpGs within the CpG island covering promoters B in normal fibroblast NHDF, NCCIT (group A), Ovcar-3 (group A), Ovcar-8 (group B) and K562 (group B) cell lines.

island in tumors revealed demethylation in testicular tumors (6/6), ovarian tumors (1/3), breast cancers (1/6) and endometrial tumors (1/3) (Table 4). Representative examples of results from studies of the cell lines and tissues are shown in Figure 6A. The partial methylation observed in normal testis tissue is likely to be due to the presence of both germ cells and non-germ cells in this tissue. As might be expected, *BORIS* was expressed in almost all the tumors and cell lines that contained a hypomethylated CpG island. Moreover, it has been shown that

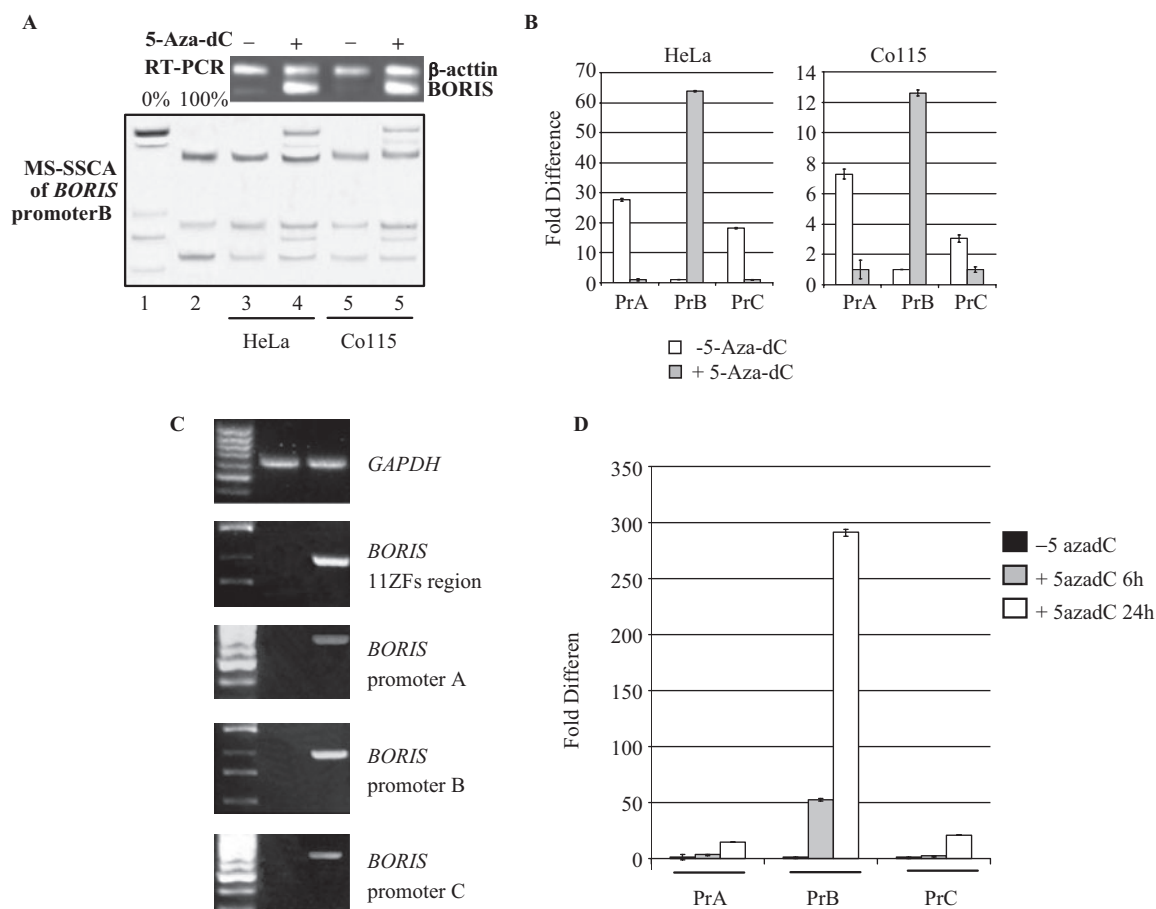
**Table 4.** *BORIS* expression and *BORIS* promoter B methylation in normal and tumor tissues

		Normal tissues	Tumor tissues
<i>BORIS</i> expression	Bladder	–	–
	Breast	–	+
	Colon	–	–
	Kidney	–	–
	Testis	+	+
	Ovary	–	+
<i>BORIS</i> promoter B methylation	Bladder	+	+
	Breast	+	– (1/6)
	Colon	+	+
	Kidney	+	+
	Testis	–	–
	Ovary	+	– (1/3)

demethylation of *BORIS* promoter B in tumor tissues, versus methylated *BORIS* promoter B in normal tissues, is correlated with the expression of *BORIS* in tumors (Table 4). However, among the 26 cell lines which belong to group A/C, only 2 showed partial demethylation of promoters B and C. Full methylation of these promoters in all the other lines was correlated with inhibition of *BORIS* transcription from promoter B (Table 1). In the case of cell lines from group B, promoters B and C were found to be unmethylated or partially methylated in the case of COLO-205. Nine out of 32 CpGs within promoters B and C were analyzed by genomic bisulfite sequencing in NHDF, 2 cell lines from group A/C (NCCIT and Ovcar-3) and 2 cell lines from group B (Ovcar-8 and K562) (Figure 6B). In normal fibroblasts, 98% of the CpGs analyzed were found to be methylated and *BORIS* expression could not be detected. In NCCIT and Ovcar-3, 98% and 96% of CpGs were methylated (Figure 6B). In these cells, *BORIS* was expressed primarily from promoter A, in association with minimal expression from promoter C and no expression from promoter B (Table 1). In Ovcar-8 and K562, sequencing showed that 0.9% and 0.45% of CpGs were methylated (Figure 6B). In these two lines, expression of *BORIS* comes almost exclusively from promoter B, 96% in Ovcar-8 and 80% in K562 (Table 1).

To confirm the regulation of promoter B by DNA methylation, we treated two weakly *BORIS*-positive cancer cell lines (HeLa and Co115) with 5-aza-dC. Treatment resulted in demethylation of the CpG island and strong activation of *BORIS* expression in both lines (Figure 7A). Moreover, the real-time PCR analyses showed that *BORIS* expression detected before 5-aza-dC treatment was coming from promoters A and C, but after 5-aza-dC treatment the *BORIS* expression switched to promoter B in both cell lines (Figure 7B). The decrease of expression from promoters A and C after 5-aza-dC treatment might be explained by the effect of 5-aza-dC itself on other transcriptional factors that could also in their turn downregulate *BORIS* expression through promoters A and C. Expression of *BORIS* in normal cells was also induced after treatment with 5-aza-dC





**Figure 7.** Effect of demethylating agent on *BORIS* expression in cancer cell lines and normal fibroblasts. **(A)** *BORIS* expression and methylation patterns of *BORIS* promoter B in two *BORIS*-negative cell lines treated with 5-aza-dC. Lanes 1–2: MS-SSCA unmethylated and fully methylated controls respectively; lane 3: HeLa cells; lane 4: HeLa cells after 5-aza-dC treatment; lane 5: Co115 cells; lane 6: Co115 cells after 5-aza-dC treatment. *BORIS* mRNA was detected by RT-PCR, with  $\beta$ -actin as internal control. **(B)** Real-time PCR on HeLa and Co115 before and after 5-aza-dC treatment. Real-time PCR data were analyzed using the comparative Ct method between treated and untreated cells and for each set of primers. **(C)** and **(D)** Determination of *BORIS* promoters A, B and C activities after treatment with 5-aza-dC of NHDFs by RT-PCR **(C)** and real-time PCR **(D)**. Real-time PCR data were analyzed using the comparative Ct method between treated and untreated cells and for each set of primers.

(Figure 7C and D) with *BORIS* transcripts originating from all three promoters. qPCR analyses showed that *BORIS* transcripts from promoter B were readily detected in cells treated for just 6 h and that transcripts from all three promoters could be detected in cells treated for 24 h with promoter B being the strongest (Figure 7D). Remarkably, we were not able to detect any demethylation of the CpG island at the 24 h time point, suggesting that a mechanism other than demethylation was responsible for the effects of 5-aza-dC on the *BORIS* promoters.

## DISCUSSION

In previous studies, it was shown that *BORIS* is normally expressed only in male germ cells where it is involved in epigenetic reprogramming (7,10). *BORIS* was also shown to be a CTA and its transcription is abnormally activated in varying proportions of a wide variety of cancers (9,25,38–40). In some cancers, *BORIS* is the most

frequently activated CTA (40). Moreover, it has been suggested that expression of *BORIS* may have diagnostic implications (39), and recent work has identified *BORIS* as an attractive candidate target for anti-cancer immunotherapy (41,42). The present study significantly extends these foundations by developing molecular understandings of the mechanisms that tightly restrict *BORIS* expression in normal tissues but are permissive for expression in many malignancies.

Here, we first identified three transcriptional start sites at –1447, –899 and –658 bp upstream of the first ATG of the *BORIS* ORF. Regions upstream of those putative transcriptional starts were tested in reporter assays and were shown to exhibit promoter activities. These three promoters designated A, B and C in association with the start sites at –1447, –899 and –658, respectively induced the synthesis of five transcripts that are different from one another in their 5'-noncoding regions. Numerous studies have documented the importance of 5'-noncoding regions for both mRNA stability (43–45) and translational

efficiency (46), as well as for having an impact on the specificity of tissue expression (47–49). Moreover, promoter regions and 5'-UTRs may determine alternative splicing of downstream exons, thus influencing the function of resulting products (50,51). We suggest that similar regulatory mechanisms might well be involved in the regulation of *BORIS* expression. In our study, we estimated the half-life of *BORIS* transcripts with alternative 5' UTRs and found that the stability of the mRNAs differed significantly. The variance ranged from 4.7 to 7.9 h, with the *BORIS* transcript having the shortest half-life and the *BORIS A3* transcript the longest. mRNA stability plays a major role in gene expression in mammalian cells, affecting the rates at which mRNAs disappear following transcriptional repression and accumulate following transcription induction (29). Thus, expression of *BORIS* alternative transcripts with different stabilities in testis and cancer cells suggests that these isoforms may have unique physiological functions, possibly related to different stages of spermatogenesis or events involved in cancer development and progression.

To understand these three promoters in greater depth, we first analyzed their utilization in a panel of normal tissues and in 31 cancer cell lines representing different types of human tumors. We demonstrated that the extent to which the promoters are employed is very different for normal testis and the cancer cell lines. In testis, all three promoters are expressed but to differing extents. Further studies will be required to determine if this pattern is common to all expressing germ cells or if promoter utilization varies depending on the state of differentiation with all being used at one point or another. In contrast, the patterns of promoter utilization in *BORIS*-positive cancer cell lines indicated that the lines fell into two main subsets: group B, comprising 16% of the lines, in which activity came primarily from promoter B; and group A/C, with 84% of the cases, in which activities come from promoters A and/or C. These results suggest that the normally stringent restriction of *BORIS* expression to testis requires repression of all three promoters in somatic cells. These data also demonstrate that release of *BORIS* expression in cancers does not reflect relaxation of promoter control to a state like that in testis but occurs in a more selective mode of promoter deregulation. Understanding if there are specific functional consequences to selective regulation of each promoter and variant transcript is a subject on ongoing studies.

As *CTCF* appears to be expressed in a complementary fashion with *BORIS* during spermatogenesis, we evaluated a potential inhibitory role for *CTCF* in *BORIS* transcription. We demonstrated that downregulation of *CTCF* by siRNA results in upregulation of *BORIS* in NHDF. We also showed that all three *BORIS* promoters contain *CTCF*-binding sites, suggesting that *CTCF* acts directly to regulate the *BORIS* promoters. The demonstration that *CTCF* is involved in the negative regulation of *BORIS* expression is likely to be important for understanding the uniform expression of *BORIS* in normal rat cells transformed due to a retroviral disruption of one *CTCF* allele (52) and in tumors

of mice heterozygous for a null allele of *CTCF* (unpublished data).

Previous research convincingly demonstrated that binding of *CTCF* to many of its targets is sensitive to CpG methylation. This is exemplified by studies of the *IGF2/H19* ICR, which showed that *CTCF* binds to the unmethylated maternal allele but not the methylated paternal allele (11–13). We thus hypothesized that regulation of *BORIS* transcription would be dependent on the methylation status of *CTCF* target sites in the promoters. Consistent with this suggestion, *BORIS* promoters B and C were found to be located in a shared CpG island. We therefore proceeded to determine how methylation status would affect the activities of these promoters. EMSAs performed using *SssI* methylated probes showed that *CTCF*-binding sites in promoter A were insensitive to methylation. In promoter B, however, *CTCF* binding is insensitive to methylation outside of the CpG island, but sensitive within the island. Likely, *CTCF* binding in promoter C was sensitive to methylation. In addition, ChIP studies showed a reverse correlation between *CTCF* binding and expression of *BORIS* from promoter B. Indeed, *CTCF* did not bind in promoter B in Ovar-8 cells that expressed *BORIS* from promoter B. In contrast, *CTCF* bound strongly in promoter B in Ovar-3 cells that expressed *BORIS* from promoter A. Analyses of methylation status of the CpG island in the Ovar-3 and BJ cell lines showed that promoters B and C were fully methylated, although *CTCF* binding was found in both promoters *in vivo*. It seems likely that these results can be explained by the limitations of ChIP resolution as promoter B also contains *CTCF* sites insensitive to methylation and promoters B and C are only ~250 bp apart. However, the possibility of indirect *CTCF* recruitment by another factor that can bind these methylated sequences cannot be formally excluded. Analyses of normal cells, tumor tissues and tumor cell lines showed that the activity of promoter B is downregulated by DNA methylation. Regulation of *BORIS* transcriptional activity by methylation was suggested but not conclusively demonstrated by other recent studies (9,53). As shown here, treatment of cancer cell lines that express *BORIS* at low levels with the demethylating agent, 5-aza-dC, resulted in demethylation of the promoter CpG island and substantial increases in *BORIS* transcription. However, treatment of *BORIS*-negative normal fibroblasts with 5-aza-dC also activated *BORIS* transcription, this time from all three promoters. The rapidity with which transcription was induced, and the lack of demethylation of the CpG island that contains promoters B and C, indicated that molecular mechanisms by which 5-aza-dC activated expression of *BORIS* in NHDF was very different from that governing activation in the tumor cells. Based on this, we conclude that although the methylation status of *BORIS* promoters is likely to be the determining regulatory feature in many cellular contexts, methylation-insensitive regulatory mechanisms exist that may well be cell type-specific.

The tumor suppressor protein, p53, is well known as a transcriptional activator of genes involved in cell cycle arrest, apoptosis, DNA repair and senescence (54,55).

Activation of transcription by p53 occurs mostly through direct binding to DNA at a consensus target site. Nevertheless, in addition to gene activation through direct binding to DNA, p53 has been shown to repress transcription by indirect interactions with a target promoter through a promoter-bound transcriptional activator as the intermediary. For example, the interaction of p53 with Sp1 inhibits *hTERT* (56), *CCNB1* (57) and *IGF1* (58) promoters, while the interaction of p53 with NF-Y inhibits *CDC2* (59), *FNI* (fibronectin) (60) and *COX2* (61) promoters. In this study, we showed that expression of wild-type p53 protein resulted in repression of all three *BORIS* promoters, but strongest forcefully for promoters A and B. However, ChIP assays showed that p53 may not bind directly to *BORIS* promoters (data not shown) or that if bound, it may be masked by multiple p53-binding co-factors from interactions with antibodies used in our assay. It is also possible that the strong inhibitory effects of p53 on *BORIS* promoters could be explained by interaction of p53 with the transcription factor Sp1 since consensus-binding sites for Sp1 are present in several copies in each *BORIS* promoter. Additionally, a recent study showed that p53 is required for the maintenance of DNA methylation patterns (62). Thus, it is possible that the maintenance of DNA methylation by p53 (62) could contribute to keeping *BORIS* silent.

In summary, we characterized three promoters for the *BORIS* gene that lead to the transcription of five mRNA differing in their 5'-UTRs. All three promoters were involved in *BORIS* expression during normal spermatogenesis; however, promoters A and C are preferentially used upon activation of *BORIS* during tumorigenesis. Interestingly, a few tumors that exhibited no activity from any of these three promoters still expressed *BORIS* transcripts (data not shown), suggesting the possible existence of additional regulatory sequences that act upstream of promoter A. Thus, DNA methylation, and expression of CTCF and p53 represent three mechanisms involved in the negative regulation of *BORIS* transcription, although it seems likely that additional levels of control also exist. These results showed that regulation of *BORIS* activity is complex, being both promoter- and cell type-dependent. Varying promoter usage driving multiple *BORIS* transcripts could influence many aspects of epigenetic reprogramming in normal development and in tumorigenesis.

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*Conflict of interest statement.* None declared.

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